Silver indol, $C_8H_6NAg.NH_3$.
Potassium carbazol, $C_{12}H_8NK.2NH_3$ and $C_{12}H_8NK.NH_3$.
Calcium carbazol, $(C_{12}H_8N)_2Ca.7NH_3$ and $(C_{12}H_8N)_2Ca.4NH_3$.
Silver carbazol, $C_{12}H_8NAg.2NH_3$ and $C_{12}H_8NAg.NH_3$.

[1] *Amer. Chem. J.*, **28**, 1902, (83); **47**, 1912, (285); *Eighth Int. Cong. App. Chem.* **6**, 1912, (119) and *J. Amer. Chem. Soc.*, **37**, 1915, (2279).

[2] Solvolysis is used as a general term to include hydrolysis, ammonolysis, amidolysis, aminolysis, alcoholysis, etc.

[3] Franklin and Kraus, *J. Amer. Chem. Soc.*, **27**, 1905, (191).

GROWTH AND REPRODUCTION IN FOWLS IN THE ABSENCE OF CAROTINOIDS AND THE PHYSIOLOGICAL RELATION OF YELLOW PIGMENTATION TO EGG LAYING

By Leroy S. Palmer

Division of Agricultural Biochemistry, University of Minnesota

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The chemical identification of each of the recognized vitamines as individual substances or chemical groups is greatly to be desired. As the result of certain studies[1] which I made on the physiological relation between the yellow carotin and xanthophyll pigments of plants and the yellow lipochromes of animal tissues and fluids, I became impressed with the fact that there seemed to be more than a casual relation between the simultaneous presence of the plant carotinoids and fat-soluble vitamine in butter fat and egg yolk and in the leafy parts of green plants, and the simultaneous absence of carotinoids and fat-soluble vitamine from lard.

That phase of my carotinoid studies showing the physiological identity of egg yolk lipochrome with plant xanthophyll suggested that the fowl should be a suitable animal upon which to test the relation of plant carotinoids to growth and reproduction. It was decided to approach the question by attempting to raise a flock of chickens from hatching to maturity on a ration devoid of carotin and xanthophylls. The problem which was presented was therefore mainly one of selecting a ration devoid of yellow plant pigments but which was presumably adequate otherwise for the normal growth of chickens.

Three experiments were undertaken. The first was a preliminary experiment, during the winter of 1916–17, to test the efficacy of a ration of white corn, white corn bran, bleached flour, skim-milk and

bone meal, beginning with birds weighing about one-half pound each. Several birds reached the period of fecundity on this ration devoid of carotinoids, and the yolks of the eggs laid were remarkably deficient in color.

In the second experiment, carried out during the season of 1917–18, 75 White Leghorn chicks were placed on a practically identical carotinoid-free ration immediately after hatching. None of these birds reached maturity, partly, it is believed, because of nutritional difficulties, and partly, it is admitted, because of a number of unfortunate accidents in caring for the birds, which caused the death of the larger share of them.

The nutritional difficulties of the second experiment were overcome in the third trial begun in April, 1918, by introducing into the ration both pork liver, which is rich in vitamines,[2] but devoid of carotinoids,[3] and a roughage of paper pulp.[4] A flock of 50 vigorous, normal White Leghorn chickens were raised from hatching to maturity on this modified white corn and skim-milk ration. The mature birds were free from yellow pigmentation.

Not only was normal growth secured on this ration, but the hens in the flock exhibited normal fecundity. Seventeen of the hens whose egg records were kept averaged 52 eggs each during a period of 233 days. Several had considerably higher records. One hen laid 44 eggs during a period of 59 days.

Especially interesting was the character of the pigmentation of the egg yolks. The hard-boiled yolks were colorless but the raw yolks had a faintly yellow color. The pigment, however, was neither carotin nor xanthophyll. Acetone readily extracted the coloring matter from the raw yolks but the pigmented fat which could be obtained from this extract failed to give the reduction test with ferric chloride, which I have shown[5] to be characteristic of carotinoids. Attempts to identify the pigment with Barbieri's[6] ovochromine or with bilirubin, were not successful.

It was felt that the negative relation between carotinoids and fat-soluble vitamine as exhibited by normal growth and fecundity in chickens on rations devoid of carotinoids could not be regarded as established unless the carotinoid-free eggs should prove fertile and normal chicks be hatched from them. Inasmuch as the cocks and hens of the carotinoid-free flock were kept together throughout the experiment the eggs were presumably fertile. About 90 eggs, in all, were incubated at various times. Forty-one livable chicks were hatched

from these eggs. The young chicks appeared normal in every way except for the complete absence of yellow pigmentation from the shanks, beaks, and other skin parts.

The newly hatched chicks were immediately placed on a carotinoid-free ration and were cared for as nearly as possible in the same manner as the young chicks of the preceding generation. By the end of three months, however, all had died. Although it is probable that this unfortunate result may be explained on the ground that the chicks were hatched very late in the season and therefore had to combat a period of extreme heat, as well as a very restricted diet during the most precarious period of their growth, nevertheless the question remains open as to whether it is possible to continue the carotinoid-free condition into more than one generation.

Physiological relation of pigmentation to egg laying.—Practical poultry men have recognized for several years that a relation exists between the amount of yellow pigment visible in the shanks, ear lobes, beak and vent of hens of the Leghorn, Plymouth Rock, Wyandotte and Rhode Island Red breeds and their previous egg laying activity. Extensive biometric analyses have been made by Blakeslee and Warner[7] and by these authors with Harris and Kirkpatrick[8] of data collected at the Storrs Agricultural Experiment Station egg laying competitions in order to establish the character of this relation. The results show a positive correlation between pale colored shanks, ear lobes, beak, etc., and a recent more or less heavy egg production.

The hypothesis which has been adopted by these investigators to explain the physiological relationship which has been observed between fecundity and pigmentation is that the growth of the egg abstracts the pigment from the body tissues. The idea that the relationship could be explained also on the basis that the egg yolk abstracts fat-soluble pigment from the food, thus precluding its localization in the body tissues, was advanced by Harris, Blakeslee and Warner[9] in an earlier paper, but was apparently abandoned. The high percentage of fat in the blood of laying hens, as compared with non-laying hens, as shown by Warner and Edmonds,[10] and by Riddle and Harris,[11] is believed by the former authors to support the hypothesis that the tissue fat is being transferred to the egg yolk during laying with a consequent subtraction of pigment.

The success attained in raising a flock of White Leghorn fowls entirely lacking in pigmentation in both adipose tissue and visible skin parts presented the opportunity for ascertaining the true physiological relation

between fecundity and the fading of the yellow pigmentation of the shanks, ear lobes, etc. The fact that the carotinoid-free hens exhibited normal fecundity enhanced greatly their value for the investigation.

The question was attacked in two ways, first, by observing the histological changes in the shank skin when carotinoid-free food was fed to non-laying pigmented birds, and second, by observing the effects on the tissue and skin pigmentation of feeding carotinoid-rich food to laying carotinoid-free hens. The birds used for the histological studies comprise several yellow shanked White Leghorn cockerels, the specific source of whose pigmentation was not known, and one cockerel from the carotinoid-free flock whose feed was changed from the carotinoid-free ration to one composed principally of yellow corn. The visible skin parts of the latter bird took on a yellow color very rapidly after the introduction of the yellow corn until at the end of 42 days his plumage had a rich creamy appearance and the shanks, beak, ear lobes and vent a deep yellow color. Each of the pigmented birds was placed on a carotinoid-free ration and histological studies made on vertical frozen sections of the shank skin of individuals from time to time as the pigment gradually faded.

As the result of these studies the observation of Barrows[12] was confirmed that the yellow pigment of the shank skin is confined chiefly to the Malphigian layer of the epidermis, with some pigment in the corium. Especially instructive were the sections after staining with Nile blue. The sample of this dye which was used was found to be dichromatic with respect to fat and pigment, fat staining red and carotinoid pigment deep blue. By this means it was determined that carotinoid pigment exists free in granular condition in the shank epidermis, which is contrary to the results reported by Barrows, who concluded that the lipochrome of the shank skin is dissolved in fat. The failure of Sudan III to color the visible skin parts of fowls, as observed by Blakeslee,[13] and confirmed by me, is explained readily by the observation that the Malphigian layer of both the pigmented and non-pigmented skin lacks appreciable amounts of stainable fat.

The histological studies of the shank skin as the xanthophyll gradually faded on the carotinoid-free ration showed first a disappearance of pigment from the corium, then a disappearance from the outer layer of the corium which gradually extended to the rete of Malphigi, the last pigment to disappear being the xanthophyll at the base of the Malphigian layer. These observations are interpreted to mean that when

the supply of xanthophyll for the skin is cut off by reason of its removal from the food, or for any other reason, any xanthophyll present in the corium layer of the skin of the shank, ear lobes, etc., is deposited in the rete of Malphigi. At the same time the xanthophyll deposits in the outer layer of the epidermis either wear off by reason of the normal replacement of the outer cells by those lower down, or are oxidized because of closer contact with the air. The xanthophyll deposits in the rete of Malphigi in time become a part of the outer layers of epidermis and are lost also. The skin thus finally becomes free from visible yellow pigment.

The significance of this interpretation at once becomes apparent in the light of the results secured when xanthophyll-rich rations were fed to the laying carotinoid-free hens. After a month on rations containing an abundance of green feed or yellow corn not a trace of xanthophyll had appeared in the ear lobes, shank or vent, and the adipose tissue had taken up such a small amount of yellow color that a very careful examination of the rendered, melted, body fat was necessary to detect the increase in color in comparison with the fat from birds which had received no carotinoids in their ration from birth. The blood serum and the yolks of the eggs laid during the feeding of the xanthophyll-rich rations, however, contained an abundance of yellow pigment.

As the result of the histological studies and feeding trials the author believes that the correct explanation of the physiological relation between egg laying and the fading of visible yellow pigmentation from the bodies of fowls of certain breeds is that in cockerels and non-laying females the visible skin parts represent a normal path of excretion of the xanthophyll pigment derived from the food. Egg laying deflects the excretion entirely to the ovaries, and even prevents the incorporation of xanthophyll with the tissue fat, and this continues as long as the ovaries function with regularity, whether the egg production be at the rate of one egg a day or one egg a week. The result is that the pigment found in the skin at the onset of fecundity is gradually excreted toward the epidermis where it either wears away as the result of the normal structural changes in the epidermis, or becomes oxidized, and thereby decolorized. The movement of yellow skin pigment during fecundity is thus outward and not inward toward the ovaries.

Influence of various feeds and certain dyes on the color of the egg yolk and body fat.—A critical study was made of the effect of certain coloring matters on the pigmentation of adipose tissue, egg yolk and visible skin parts, and also of the relative xanthophyll content of various

materials commonly used as chicken feed, using the carotinoid-free flock.

Carotin alone, fed as naturally highly colored butter fat, and the orange-yellow pigment of the annatto seed, were found to be without influence on the color of the adipose tissue or visible skin parts. Sudan III colored only the adipose tissue of non-laying birds, the visible skin parts being unaffected by this dye. With laying birds, the egg yolk as well as the adipose tissue was colored by Sudan III, but not the visible skin parts.

Yellow corn and green feed only were found to be rich in xanthophyll when various plant and animal materials were tested for their xanthophyll content by their effect on the color of the egg yolks when fed to hens laying carotinoid-free eggs. A little of the pigment was found in hempseed, barley, gluten feed and red corn. Wheat, wheat bran, oats, cottonseed meal, rape seed, meat scraps, blood meal, skim-milk and butter-milk were found to contain negligible quantities of xanthophyll.

The experiments here reviewed were conducted at the Missouri Agricultural Experiment Station with the coöperation of Prof. H. L. Kempster of the Department of Poultry Husbandry. The complete data appeared in a recent issue of the *Journal of Biological Chemistry*.

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